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## **Oropharyngeal group A streptococcal colonization disrupts latent epstein-barr virus infection**

Ueda, Seigo ; Uchiyama, Satoshi ; Azzi, Tarik ; Gysin, Claudine ; Berger, Christoph ; Bernasconi, Michele ; Harabuchi, Yasuaki ; Zinkernagel, Annelies S ; Nadal, David

**Abstract:** Epstein-Barr virus (EBV) infects >90% of the human population within the first 2 decades of life and establishes reversible latent infection in B cells. The stimuli that lead to switching from latent to lytic EBV infection in vivo are still elusive. Group A streptococci (GAS) are a common cause of bacterial pharyngotonsillitis in children and adolescents and colonize the tonsils and pharynx of up to 20% of healthy children. Thus, concomitant presence of EBV and GAS in the same individual is frequent. Here, we show that EBV carriers who are colonized with GAS shed EBV particles in higher numbers in their saliva, compared with EBV carriers not colonized with GAS. Messenger RNA levels of the master lytic regulatory EBV gene BZLF1 were more frequently detected in tonsils from EBV carriers colonized with GAS than from EBV carriers not colonized. Heat-killed GAS, potentially mimicking GAS colonization, elicited lytic EBV in latently infected lymphoblastoid cell lines (LCLs) partially via Toll-like receptor 2 triggering, as did purified GAS peptidoglycan. Thus, colonization by GAS might benefit EBV by increasing the EBV load in saliva and thereby enhancing the likelihood of EBV spread to other hosts.

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# Oropharyngeal Group A Streptococcal Colonization Disrupts Latent Epstein-Barr Virus Infection

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Epstein-Barr virus (EBV) infects >90% of the human population within the first 2 decades of life and establishes reversible latent infection in B cells. The stimuli that lead to switching from latent to lytic EBV infection in vivo are still elusive. Group A streptococci (GAS) are a common cause of bacterial pharyngotonsillitis in children and adolescents and colonize the tonsils and pharynx of up to 20% of healthy children. Thus, concomitant presence of EBV and GAS in the same individual is frequent. Here, we show that EBV carriers who are colonized with GAS shed EBV particles in higher numbers in their saliva, compared with EBV carriers not colonized with GAS. Messenger RNA levels of the master lytic regulatory EBV gene *BZLF1* were more frequently detected in tonsils from EBV carriers colonized with GAS than from EBV carriers not colonized. Heat-killed GAS, potentially mimicking GAS colonization, elicited lytic EBV in latently infected lymphoblastoid cell lines (LCLs) partially via Toll-like receptor 2 triggering, as did purified GAS peptidoglycan. Thus, colonization by GAS might benefit EBV by increasing the EBV load in saliva and thereby enhancing the likelihood of EBV spread to other hosts.

**Keywords.** Epstein-Barr virus (EBV); group A *Streptococci* (GAS); tonsil; oropharynx; lytic; latent; TLR2; salivary shedding.

More than 90% of the adult population is persistently and asymptomatically infected with Epstein-Barr virus (EBV), a human B-lymphotropic  $\gamma$ -herpesvirus [1]. Primary infection with EBV is acquired mainly in childhood via saliva [1]. The oropharynx is the portal of entry and exit, where the palatine tonsils act as reservoir for EBV [2–4]. In its latent form in B cells, EBV expresses a limited number of genes, does not replicate, and its DNA is propagated to daughter cells during cell division. By contrast, in its lytic form, EBV expresses

genes required for replication and generation of infectious virus particles resulting in host cell lysis and virus release [5]. The default state of EBV infection is the latent form, which is reversible, permitting the creation of new viral particles and transmission to other hosts [6]. The mechanisms responsible for switching to the lytic form in vivo are not completely understood.

EBV is associated with Burkitt lymphoma (BL), Hodgkin disease (HD), and posttransplantation lymphoproliferative disease [7]. These B-cell tumors display distinct patterns of EBV latency gene expression [8]. The oncologic potential of latent EBV is indicated by its unique capacity to growth transform B cells in vitro to lymphoblastoid cell lines (LCLs) [1]. Thus, disruption of latency is not only essential to enable transmission of EBV to other hosts but might be an important factor to limit EBV-induced B-cell lymphoproliferation.

Being immunocompromised increases the risk of EBV-associated B-cell tumors [9]. But nonimmunocompromised individuals may also develop EBV-associated

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BL or HD. EBV-associated HD is more likely to develop when primary EBV infection occurs in adolescence and manifests as infectious mononucleosis with an exuberant immune activation [10]. In endemic BL, >95% of the tumors are EBV positive, and they are epidemiologically linked to *Plasmodium falciparum* malaria, resulting in chronic immune activation [11]. The pattern-recognition receptor Toll-like receptor 9 (TLR9) is abundantly expressed in B cells, and it senses DNA and the malaria parasite's pigment hemozoin [12–14]. We recently demonstrated that TLR9 activation of B cells inhibits lytic EBV during primary infection and inhibits the switching of latent to lytic EBV in chronic infection in vitro [15, 16].

Group A streptococci (GAS) colonize tonsils and the pharynx of up to 20% of healthy children [17]. Considering the high prevalence of EBV and GAS, concomitant presence of both microorganisms in the same individual is frequent. TLR9 senses bacterial DNA and is crucial for controlling GAS infections [18]. One may thus reason that the presence of GAS in tonsils may direct the EBV life cycle toward latency, thereby impairing EBV transmission to other hosts via saliva.

Here, we investigated the influence of GAS on EBV's life cycle and salivary shedding and the mechanisms involved.

## METHODS

### Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The Ethics Commission of the Canton of Zurich approved the study (StV 40/05). All subjects or their caregivers provided written informed consent.

### Cell Culture

The EBV-producer cell line B95.8, Akata BL cells, tonsillar mononuclear cells (TMCs), and LCLs were maintained in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich, Buchs, Switzerland) with 10% heat-inactivated fetal bovine serum (Life Technologies, Zug, Switzerland), 1% L-glutamine, and 1% penicillin-streptomycin, referred to hereafter as R10.

### Enzyme-Linked Immunosorbent Assay (ELISA)

Interleukin 6 (IL-6) or interleukin 10 (IL-10) levels were measured using human IL-6 or IL-10 ELISA kits (R&D Systems, Abingdon, United Kingdom) following the manufacturer's instructions.

### DNA Extraction and EBV DNA Detection

Saliva samples were obtained and DNA was extracted as reported elsewhere [19]. EBV DNA levels were determined by quantitative real-time PCR (qPCR) targeting the conserved EBV *BamHI* W region, as reported elsewhere [20].

### RNA Extraction and qPCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. After DNase I treatment, 1 µg of total RNA was used as template for reverse transcription by use of a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). qPCR for human and EBV gene messenger RNA (mRNA) was performed using specific primers and probes for *IL-6*, *IL-10*, or *BZLF1*, as reported elsewhere [15], and for *TLR1–10* (Life Technologies). All reactions were performed on a real-time PCR machine (7900HT; Life Technologies) with TaqMan Gene Expression Master Mix (Life Technologies). The relative gene expression was calculated for each gene of interest by using a  $\Delta\Delta C_T$  method, where  $C_T$  values were normalized to the value for the housekeeping gene hydroxymethylbilane synthase (*HMBS*) [15].

### Isolation of TMCs and EBV Serology

TMCs were isolated from palatine tonsils obtained from patients who underwent routine tonsillectomy, as reported elsewhere [21]. The EBV serologic characteristics of the TMC donors were determined using the Immunodot-Mono G and Mono M kit according to the manufacturer's instructions (Ruwig Diagnostics, Bettlach, Switzerland).

### Preparation of Stock EBV

Supernatant of B95.8 cells was obtained as reported elsewhere [21] and stored at  $-80^{\circ}\text{C}$ . The cell-free supernatants contained approximately  $1 \times 10^8$ /mL EBV DNA as evaluated by qPCR [20].

### GAS Strains

The well-characterized clinical isolate M1T1 GAS strain 5448 [22] was grown to logarithmic phase in Todd-Hewitt broth (Becton Dickinson, Allschwil, Switzerland) containing 2% yeast extract (THY; Oxoid, Pratteln, Switzerland) and was resuspended in Roswell Park Memorial Institute 1640 medium at a final concentration of  $2 \times 10^9$  colony-forming units/mL. Bacteria were killed by heating (at  $85^{\circ}\text{C}$  for 60 minutes) or by sonication (Sonoplus HD2070; Bandelin Electronic, Berlin, Germany) with 20 kHz at 70 W (amplitude, 100%) for 15 minutes on ice.

### Detection of GAS Colonization

Explanted tonsils were rolled over 5% sheep blood agar that was incubated for 48 hours. GAS was identified by latex agglutination for Lancefield group A (Bio-Rad, Cressier, Switzerland) and detection of pyrrolidonyl peptidase production, using L-pyrrolidonyl-beta-naphthylamid (0.7%) disks (Oxoid, Pratteln, Switzerland).

### Stimulation of EBV-Infected Cells With GAS

To model acute infection, TMCs plus B95.8 culture supernatants were used, and to model persistent infection, LCLs and Akata cells [23] were used. LCLs were established from TMCs

by infection with B95.8 EBV. Heat-killed or sonicated GAS at a multiplicity of infection (MOI) of 100 or 20  $\mu\text{g/mL}$  of GAS peptidoglycan (Toxin Technology, Sarasota, FL) was added for 24 hours. Apoptosis was assessed using a PE Annexin V Apoptosis Detection Kit I according to the manufacturer's instructions (Becton Dickinson). To neutralize TLR2, LCLs were preincubated with anti-TLR2 polyclonal antibodies (LabForce AG-In vivoGen, Nunningen, Switzerland).

### Flow Cytometry

For TLR2 detection, fluorescein isothiocyanate (FITC)-labeled anti-TLR2 monoclonal antibodies (TL2.1; LabForce AG-In vivoGen) were used, and FITC-labeled mouse immunoglobulin G2a (Becton Dickinson) was used as isotype control. For TLR2 and BZLF1 detection, following TLR2 staining, cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) and permeabilized using 0.4% Triton X-100 before adding anti-BZLF1 antibodies (BZ1; Santa Cruz Biotechnology, Heidelberg, Germany) followed by PE-labeled rabbit anti-mouse immunoglobulin G1 (Santa Cruz Biotechnology). Data on apoptosis or TLR2 and/or BZLF1 detection were collected using a FACSCanto II (Becton Dickinson) and were analyzed using FlowJo software.

### Statistical Analyses

Analyses of statistical significance were based on a 2-tailed paired or unpaired Student *t* test, Wilcoxon signed rank test, Mann-Whitney *U* test, or  $\chi^2$  test. Differences with a *P* value of  $<.05$  were regarded as statistically significant.

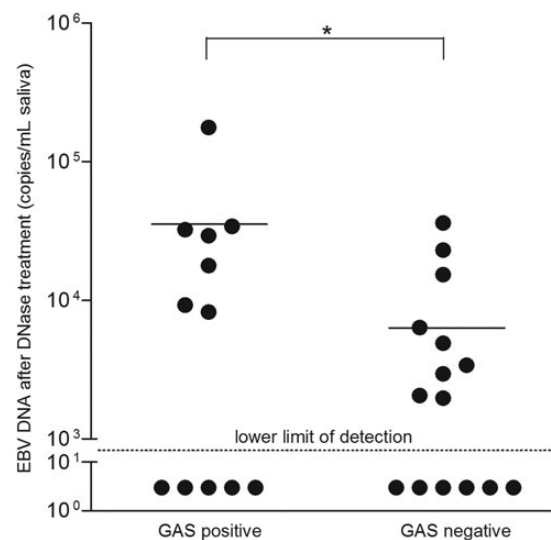
## RESULTS

### Saliva From EBV-Infected GAS-Colonized Individuals Contained More EBV Than Saliva From EBV-Infected Individuals Not Colonized With GAS

We asked whether salivary EBV shedding is influenced by colonization with GAS. Thus, we assayed saliva from EBV-infected individuals who were or were not colonized with GAS by using qPCR targeting EBV DNA to detect viral DNA contained in intact EBV particles. We detected EBV in 7 of 12 GAS-colonized individuals (58%; median age, 6.0 years [range, 2.2–15.0 years]; mean age, 6.2 years) and 9 of 15 GAS-negative individuals (60%; median age, 5.3 years [range, 3.1–13.8 years]; mean age, 6.0 years). The number of EBV DNA copies in saliva from GAS-colonized EBV-infected individuals was higher than that in saliva from noncolonized individuals ( $P = .03$ ; Figure 1).

### More-Frequent BZLF1 mRNA Expression in Tonsils From GAS-Colonized Than From Noncolonized EBV-Infected Individuals

The EBV load in saliva may reflect distinct EBV replication in oropharyngeal epithelial cells, mucosa-associated B cells, or both. Since determination of EBV replication in primary epithelial cells is not feasible, we measured *BZLF1* mRNA expression in tonsils. *BZLF1* is an immediate-early lytic EBV gene,

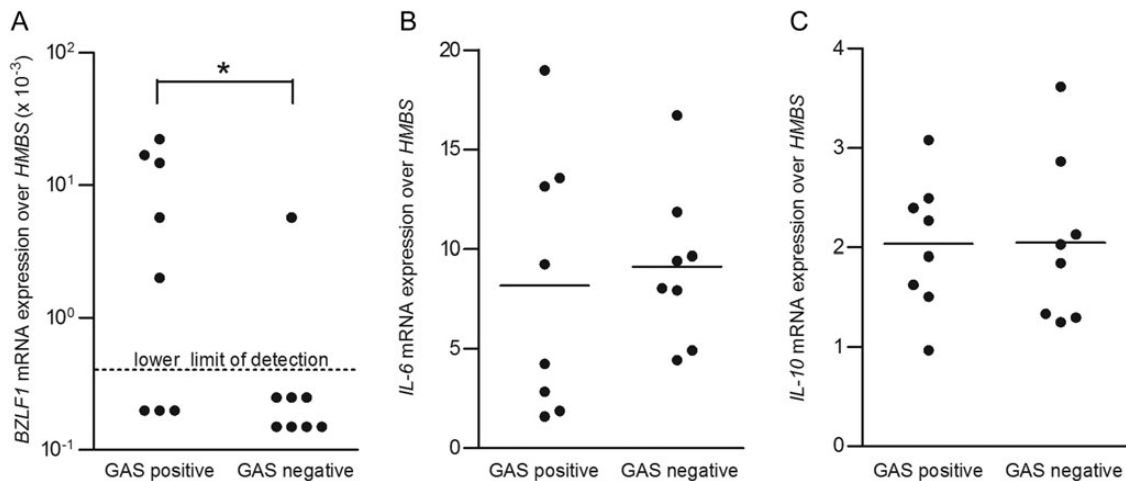


**Figure 1.** Saliva from Epstein-Barr virus (EBV)-infected group A streptococcus (GAS)-colonized individuals contained more EBV particles than saliva from EBV-infected noncolonized individuals. The number of EBV DNA copies per milliliter in saliva samples from EBV carriers who underwent routine tonsillectomy was determined by quantitative real-time polymerase chain reaction after DNase I treatment. Results for GAS-positive ( $n = 12$ ) and GAS-negative ( $n = 15$ ) individuals were compared. *P* values were calculated using the Mann-Whitney *U* test.  $*P = .03$ .

the expression of which is sufficient to initiate EBV replication. Tonsil tissues from 5 of 8 GAS-colonized individuals expressed *BZLF1* mRNA (62.5%; median age, 5.3 years [range, 2.8–6.8 years]; mean age, 4.9 years), in contrast to 1 of 8 GAS-negative individuals (12.5%; median age, 4.8 years [range, 3.1–6.0 years]; mean age, 4.8 years;  $P = .04$ ; Figure 2A). IL-6 and IL-10 mRNA expression levels determined by human specific qPCRs showed no significant differences between GAS-positive and GAS-negative tonsils (Figure 2B and 2C), suggesting a similar inflammation status.

### Heat-Killed but Not Sonicated GAS Induced Lytic EBV In Vitro After EBV Exposure

Heat-killed GAS are intact bacteria that mimic colonizing GAS. In contrast, sonication disrupts GAS, resulting in fragmentation and release of bacterial cell wall components and DNA, mimicking an acute GAS infection during which GAS are lysed by both the immune system and antibiotics. We inoculated TMCs with EBV B95.8 and concomitantly stimulated them with heat-killed or sonicated GAS at a MOI of 100. We then measured EBV and *HMBS* mRNA expression levels by qPCR 24 hours after inoculation with EBV. Levels of *BZLF1* mRNA in TMCs 24 hours after stimulation with heat-killed GAS were higher than in nonstimulated TMCs or in TMCs stimulated with sonicated GAS ( $P = .03$ ; Figure 3A). Furthermore, heat-killed GAS did not affect latent EBV (LMP1 and EBNA3C; data not

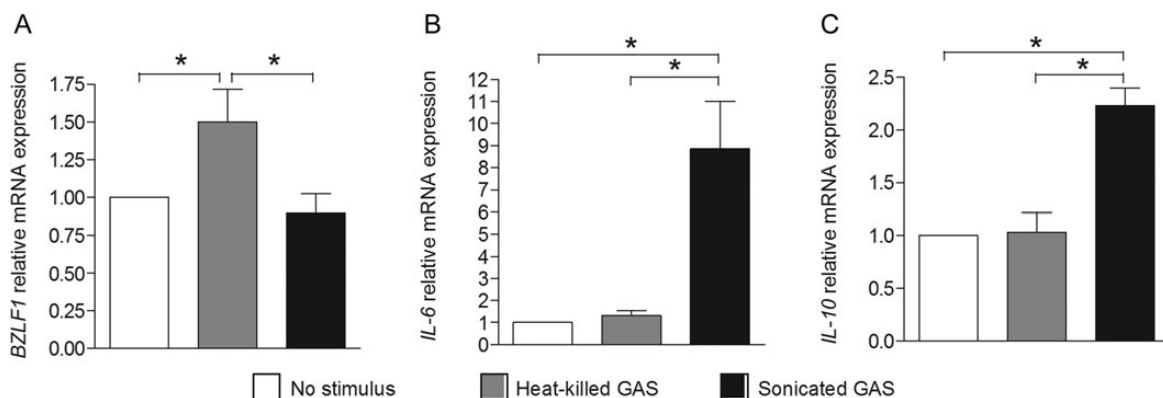


**Figure 2.** *BZLF1* messenger RNA (mRNA) in tonsils from Epstein-Barr virus (EBV)-infected group A streptococcus (GAS)-colonized individuals was more frequently detectable than in noncolonized individuals. Levels of *BZLF1* (A), *IL-6* (B), and *IL-10* (C) mRNA expression in tonsil tissues from EBV-seropositive patients who underwent routine tonsillectomy were analyzed by quantitative real-time polymerase chain reaction. Results for GAS-positive ( $n=8$ ) and GAS-negative ( $n=8$ ) individuals were compared. *BZLF1* expression values above the lower detection limit were considered positive, and those below the lower detection limit were considered negative.  $P$  values were calculated using the  $\chi^2$  test.  $*P=.04$ .

shown), IL-6, and IL-10 mRNA expression in TMCs, compared with no stimulus (Figure 3B and 3C). In contrast, sonicated GAS increased IL-6 and IL-10 mRNA expressions in TMCs, compared with no stimulus or heat-killed GAS ( $P=.03$ ; Figure 3B and 3C). Thus, neither heat-killed nor sonicated GAS influenced latent EBV gene expression. In contrast, heat-killed GAS strongly induced lytic EBV infection in TMCs exposed de novo to EBV, whereas sonicated GAS did not. The latter induced strong cytokine gene expression in TMCs, whereas heat-killed GAS did not.

#### Heat-Killed GAS Induced Lytic EBV and Caused Cell Death in Latently EBV-Infected B Cells

Next, we asked whether GAS modulates EBV persistent B-cellular infection. We used LCLs as a model for persistent (latent) EBV infection and stimulated them with heat-killed or sonicated GAS at a MOI of 100. EBV and mRNA expression levels of human genes were measured by qPCR 24 hours later. *BZLF1* mRNA levels in LCLs exposed to heat-killed GAS were higher ( $P=.02$ ) than in LCLs with or without exposure to sonicated GAS (Figure 4A).



**Figure 3.** Heat-killed but not sonicated group A streptococcus (GAS) induced lytic Epstein-Barr virus (EBV) after de novo in vitro EBV exposure. Tonsillar mononuclear cells (TMCs) were inoculated with supernatants from B95.8 cells (EBV-producer cells). Concomitantly, heat-killed or sonicated GAS were added, and then cell pellets were harvested and subjected to quantitative real-time polymerase chain reaction to determine expression of *BZLF1* (A), *IL-6* (B), and *IL-10* (C) messenger RNA (mRNA) expression. Results shown were pooled from TMCs from 6 donors and are expressed as mean values  $\pm$  standard error of the mean.  $P$  values were calculated using a Wilcoxon signed rank test.  $*P=.03$ .



We asked whether the increased *BZLF1* mRNA levels after exposure to heat-killed GAS were associated with B-cell death. We assessed late apoptosis by determining the percentage of 7-aminoactinomycin D (7-AAD)-stained cells by flow cytometry in LCLs following stimulation or no stimulation. Exposure to heat-killed GAS for 24 hours resulted in higher percentages of 7-AAD-stained cells as compared to exposure to sonicated GAS or controls ( $P = .008$ ; Figure 4B). There were no differences in percentages of early apoptotic cells as assessed by flow cytometry after staining with Annexin V and 7-AAD. This was confirmed by assessing cell viability, using the Trypan blue exclusion method (not shown). The number of EBV DNA copies after DNase I treatment in the supernatants of LCLs exposed to heat-killed GAS were higher than in controls ( $P = .02$ ; Figure 4C), corroborating our above-described in vivo observations of higher EBV copies in tonsils of GAS-colonized individuals. Exposure to sonicated GAS resulted in lower EBV DNA copies, compared with no exposure ( $P = .02$ ; Figure 4C). This is in line with our previous study showing that TLR9 triggering results in suppression of switching from latent to lytic EBV. Sonicated GAS contain increased levels of GAS DNA, which has been recently demonstrated to trigger TLR9 and subsequently induce proinflammatory cytokine expression [18, 24].

Heat-killed GAS did not affect the levels of *EBNA3C* latent EBV (not shown) or *IL-6* (Figure 4D), although it slightly upregulated *IL-10* mRNA expression (Figure 4E). Similarly, after de novo EBV exposure, sonicated GAS increased *IL-6* ( $P = .02$ ; Figure 4D) and *IL-10* mRNA expression in LCLs, compared with no stimulus ( $P = .03$ ; Figure 4E), as well as *IL-6* and *IL-10* protein levels (Figure 4F and 4G).

We wondered whether heat-killed or sonicated GAS exhibited similar effects on EBV-carrying BL cells as on LCLs. We chose Akata cells because they represent a well-established model for studying the switching from latent to lytic EBV in vitro. We analyzed *BZLF1* mRNA expression 24 hours after treatment with heat-killed or sonicated GAS by qPCR. In contrast to LCLs, levels of *BZLF1* mRNA in Akata cells exposed to heat-killed GAS were not altered, whereas exposure to sonicated GAS significantly decreased endogenous levels of *BZLF1* mRNA expression (Figure 4H). These results suggested that sonicated GAS might trigger TLR9. Sonicated GAS samples contain genomic DNA with unmethylated CpG motifs known to trigger TLR9 [18, 24], and triggering of TLR9 results in suppression of lytic induction in Akata cells [15, 16]. No difference in the percentage of 7-AAD stained cells was observed in the cells exposed to heat-killed or sonicated GAS (Figure 4I).

#### Primary B Cells and LCLs Expressed TLR2, in Contrast to Akata Cells

Gram-positive bacteria, such as GAS, are recognized by TLR2 on the cell surface and by TLR9 in endosomes [18, 24]. TLRs

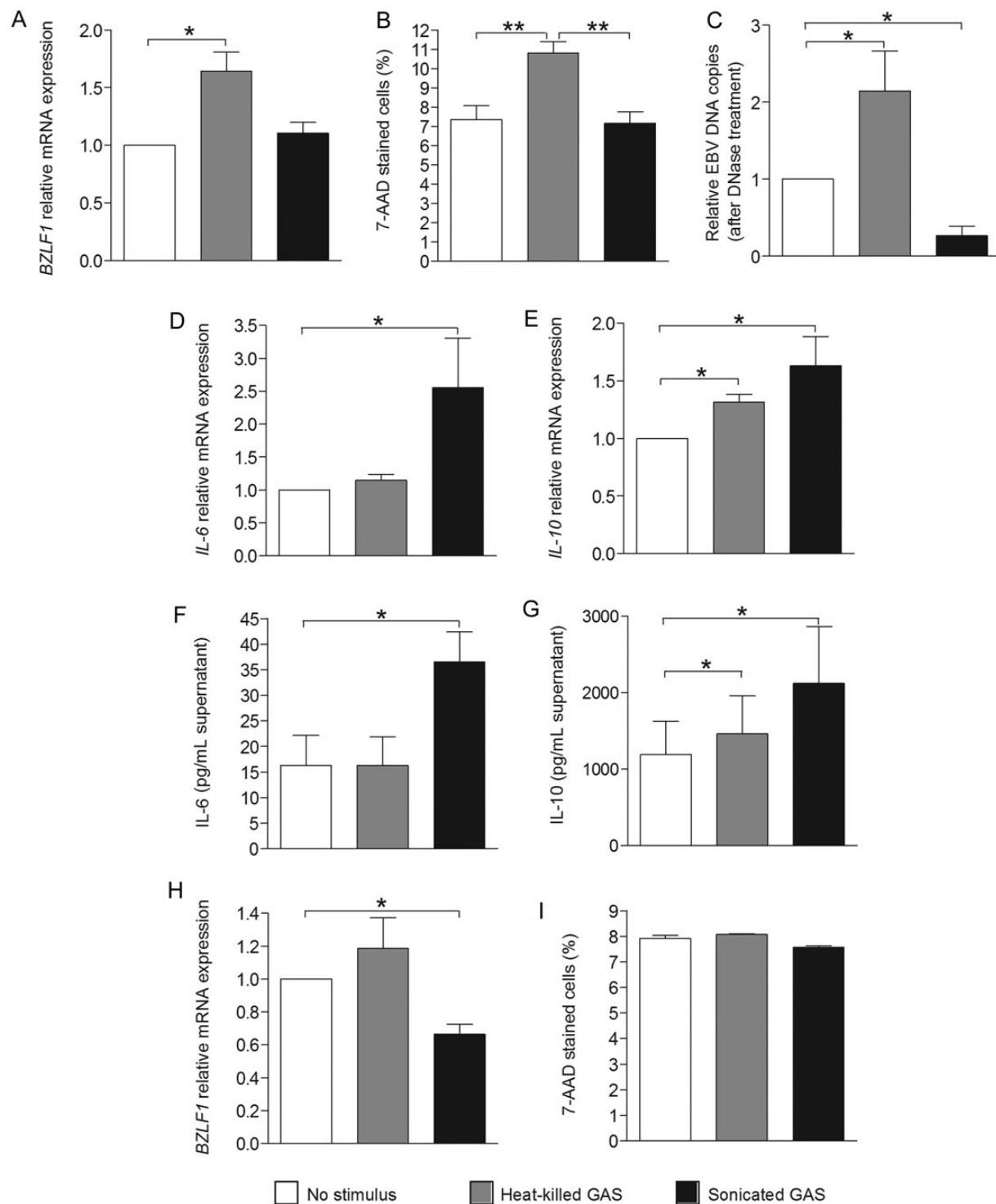
are key players in innate immunity and are involved in the recognition of pathogens and microbial products leading to activation of antimicrobial effector pathways [25]. TLR2 generally forms heterodimers with TLR1 or TLR6, which recognize peptidoglycan and lipoteichoic acids from gram-positive bacteria [26]. We asked whether the differences in lytic EBV gene expression were due to distinct expression profiles of TLRs. Thus, we analyzed TLR expression in primary B cells, LCLs, and Akata cells (Figure 5A). Primary B cells, LCLs, and Akata cells expressed *TLR1*, *TLR6*, *TLR7*, *TLR9*, and *TLR10* mRNA, whereas *TLR5* mRNA expression was detected at extremely low levels in primary B cells and was not detected in LCLs and Akata cells. *TLR2*, *TLR3*, and *TLR4* mRNA was detected at extremely low levels in Akata cells, in contrast to primary B cells and LCLs. Similar to *TLR2* mRNA, TLR2 protein was expressed in LCLs but not in Akata cells (Figure 5B), and the mean fluorescence intensity on LCLs was higher than on Akata cells (Figure 5C), as determined by flow cytometry. Since heat-killed Gram-positive bacteria are known TLR2 ligands [27] above results suggested that heat-killed GAS might induce lytic EBV infection in LCLs via TLR2 activation.

#### Heat-Killed GAS and GAS Peptidoglycan Induce Lytic EBV via TLR2

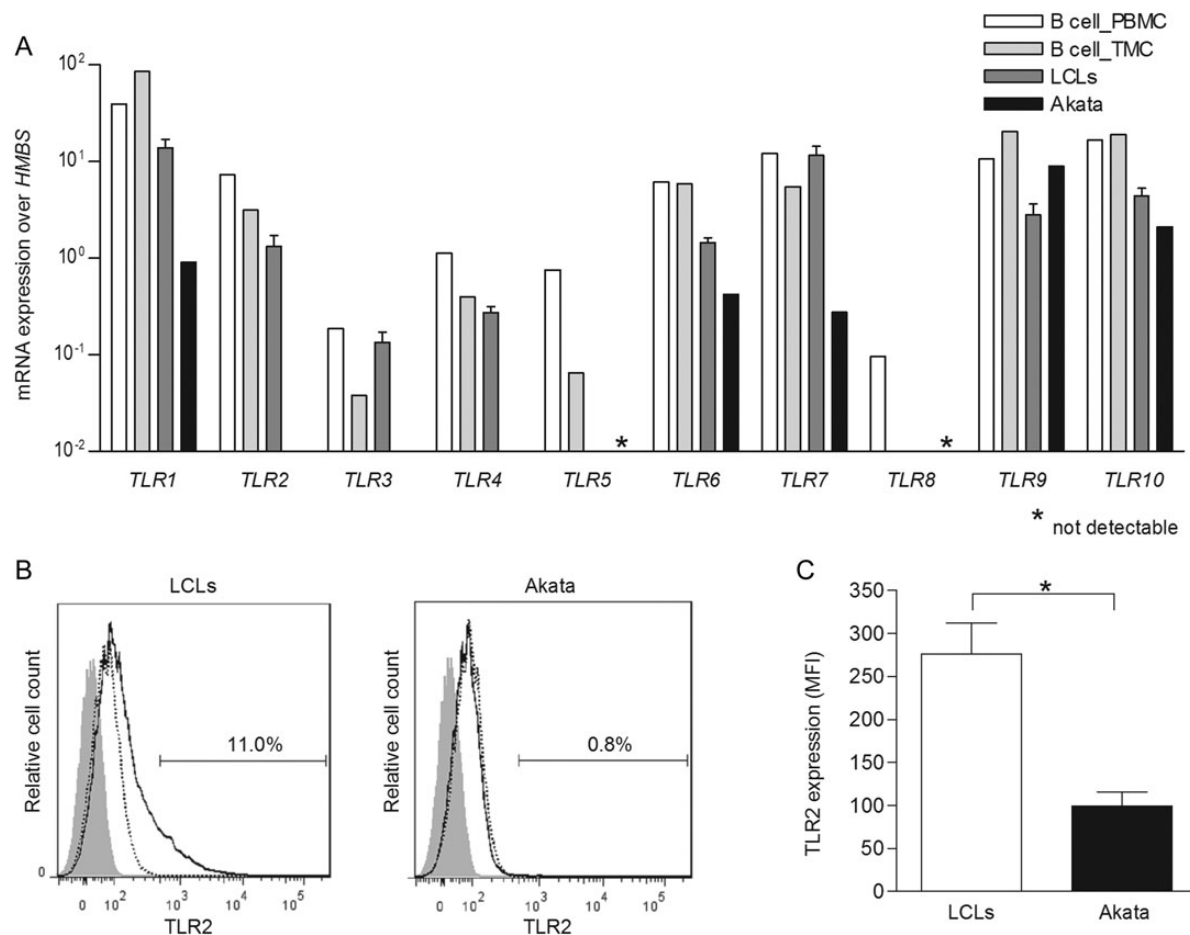
Exposure of LCLs to either heat-killed GAS, purified GAS peptidoglycan used as control for the GAS cell wall component peptidoglycan, or sonicated GAS did not change the percentages of TLR2-positive cells as determined by flow cytometry (Figure 6A and 6B upper panel left). Percentages of *BZLF1*-positive cells in LCLs exposed to heat-killed GAS were higher than in nonexposed LCLs or in LCLs exposed to sonicated GAS (Figure 6A and 6B), corroborating mRNA expression findings (Figure 4A), and exposure to GAS peptidoglycan showed results similar to those for the heat-killed GAS. The proportions of *BZLF1*-positive cells showed around a 4-fold higher increase among TLR2-positive cells, compared with TLR2-negative cells, after exposure to heat-killed GAS or GAS peptidoglycan (Figure 6B). We confirmed that exposure of LCLs to GAS peptidoglycan resulted in similar upregulation of *BZLF1* and *IL-10* mRNA as heat-killed GAS, compared with no exposure (Figure 6C). Similar results were obtained using 2 other TLR2 ligands, staphylococcal lipoteichoic acid and staphylococcal peptidoglycan (Supplementary Figure 1) [28, 29]. Finally, anti-TLR2 polyclonal neutralizing antibodies inhibited upregulation of *BZLF1* and *IL-10* mRNA expression (Figure 6C) in LCLs exposed to heat-killed GAS or GAS peptidoglycan.

## DISCUSSION

We examined the influence of GAS oropharyngeal colonization on EBV's life cycle. We found that (1) EBV carriers shed more EBV particles in their saliva when colonized with GAS, (2)



**Figure 4.** Heat-killed group A streptococcus (GAS) induced lytic Epstein-Barr virus (EBV) and caused increased cell death of latently EBV-infected B cells. Levels of *BZLF1* (A), *IL-6* (D), and *IL-10* (E) messenger RNA (mRNA) expression in lymphoblastoid cell lines (LCLs) were analyzed by quantitative real-time polymerase chain reaction (qPCR) 24 hours after exposure. B, 7-aminoactinomycin D (7-AAD)-stained LCLs were analyzed by flow cytometry 24 hours after exposure. The numbers of EBV DNA copies in culture supernatants from LCLs were analyzed by qPCR 24 hours after exposure (C). Interleukin 6 (IL-6; F) and interleukin 10 (IL-10; G) concentrations in supernatants from LCLs were measured by enzyme-linked immunosorbent assay (ELISA). The results shown were pooled from LCLs from 7 donors, from 4 donors (done in duplicate), and from 6 donors for qPCR, flow cytometry, and ELISA, respectively, and are expressed as mean values  $\pm$  standard error of the mean (SEM). *P* values were calculated by the Wilcoxon signed rank test. \**P* < .05, \*\**P* < .01. H, Levels of *BZLF1* mRNA expression in Akata Burkitt lymphoma (BL) cells were analyzed by qPCR 24 hours after exposure to heat-killed or sonicated GAS. I, 7-AAD-stained Akata BL cells were analyzed by flow cytometry 24 hours after exposure. Results shown were from Akata BL cells pooled from 3 experiments and are expressed as mean values  $\pm$  SEM. The *P* value was calculated using a paired Student *t* test. \**P* < .05.



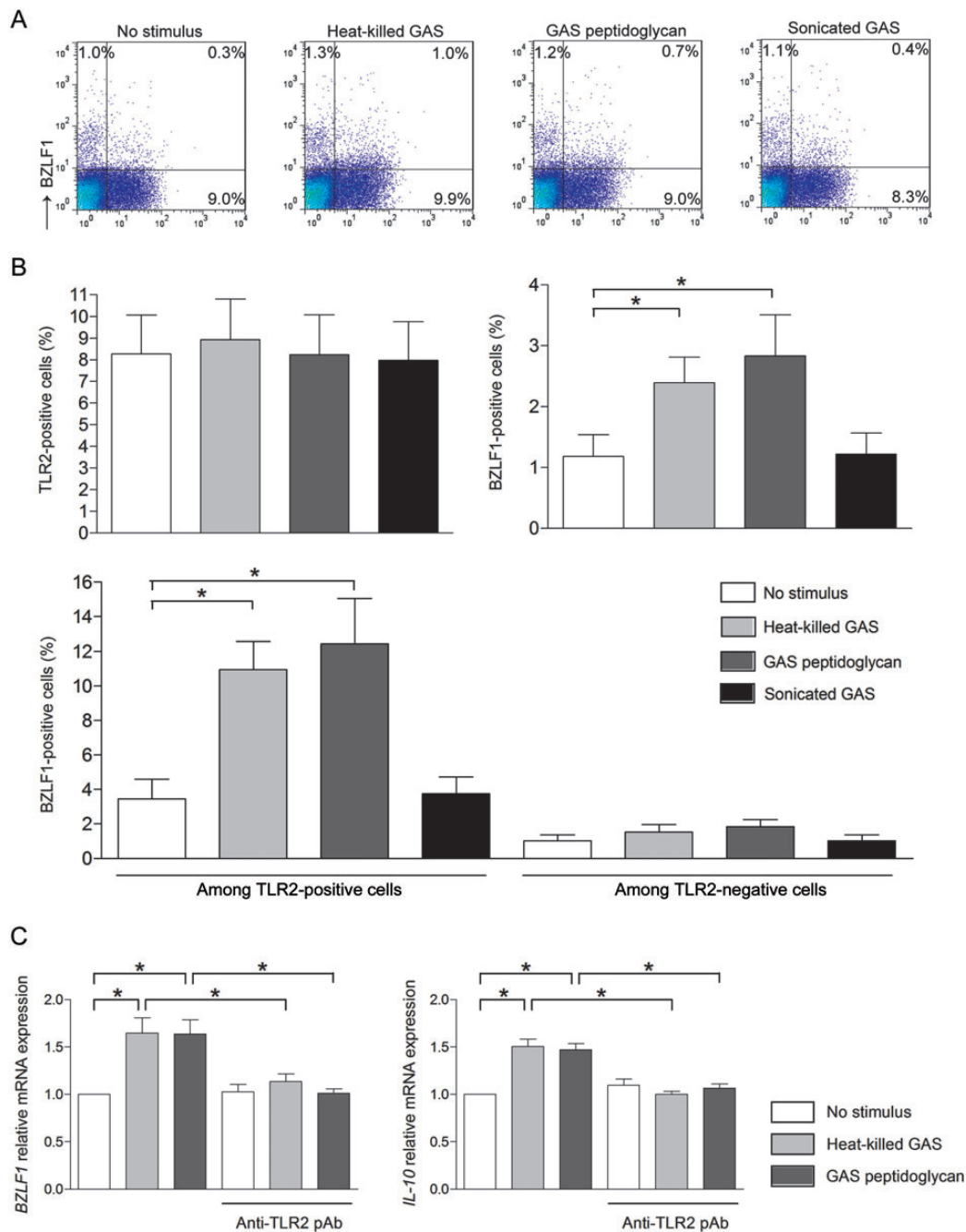
**Figure 5.** Primary B cells and lymphoblastoid cell lines (LCLs) expressed Toll-like receptor 2 (TLR2), in contrast to Akata BL cells. *A*, Messenger RNA (mRNA) expression levels of *TLR1–TLR10* in peripheral blood and tonsillar primary B cells, LCLs ( $n=4$ ), or Akata BL cells were analyzed by quantitative real-time polymerase chain reaction. Representative data of flow cytometry for TLR2 on LCLs and Akata BL cells (*B*) and the mean fluorescence intensity (MFI) from Akata BL cells pooled from 3 experiments and LCLs from 3 donors (*C*) are shown. The closed histogram, dotted line, and solid line represent unstained cells, isotype control, and anti-TLR2 antibody, respectively. The  $P$  value was calculated using an unpaired Student  $t$  test.  $*P<.05$ .

EBV's immediate-early lytic gene *BZLF1* mRNA expression was more frequent in tonsils from GAS-positive EBV carriers than from GAS-negative EBV carriers, (3) heat-killed GAS induced lytic EBV in tonsils during primary infection with EBV in vitro and in persistently EBV-infected LCLs resulting in cell death, and (4) heat-killed GAS and purified GAS peptidoglycan induced lytic EBV in LCLs via TLR2 activation. Thus, GAS colonization of the oropharynx might benefit EBV by increasing the salivary EBV load and thereby enhancing the likelihood of EBV spread to other hosts.

Our observation that concomitant GAS colonization resulted in higher salivary EBV shedding is unprecedented. Salivary EBV shedding lasts at least 6 months after primary EBV infection manifesting as infectious mononucleosis [10, 30, 31]. Frequent and abundant salivary EBV shedding is observed in EBV-infected children with tonsillar hypertrophy [19], a

condition that is associated with more-frequent GAS colonization [32]. Lytic EBV replication in the oropharynx may occur in epithelial cells, which act as amplifiers [33] after acquiring EBV from tonsillar or other local mucosa-associated B cells [34]. Investigation of lytic EBV replication in oropharyngeal epithelial cells has not been successful. Thus, we assessed the effect of GAS carriage in tonsils from EBV carriers. We found that GAS carriage was associated with significantly higher immediate-early lytic gene *BZLF1* mRNA expression, which induces switching from latent to lytic EBV [1, 6, 35]. In vitro, upregulation of *BZLF1* is induced by sodium butyrate [36], 12-O-tetradecanoylphorbol-13-acetate [37], cross-linking of B-cell receptor [38, 39], or the antiinflammatory cytokine transforming growth factor  $\beta$  [40]. Based on IL-6 and IL-10 expression, the inflammation status of tonsils from GAS-colonized individuals did not differ from that of tonsils from noncolonized





**Figure 6.** Heat-killed group A streptococcus (GAS) and GAS peptidoglycan induced lytic Epstein-Barr virus via Toll-like receptor 2 (TLR2). *A*, Representative flow cytometry findings for TLR2 and BZLF1 in lymphoblastoid cell lines (LCLs) unstimulated or simulated with heat-killed GAS, GAS peptidoglycan, or sonicated GAS. *B*, Percentages of TLR2-positive cells, BZLF1-positive cells, and BZLF1-positive cells among TLR2-positive cells and TLR2-negative cells following no treatment or treatment with heat-killed GAS or GAS peptidoglycan. *C*, Levels of *BZLF1* and *IL-10* RNA expression in LCLs following no exposure or exposure to heat-killed GAS, GAS peptidoglycan, or sonicated GAS without or with antecedent anti-TLR2 polyclonal neutralizing antibodies (anti-TLR2 pAb) treatment were analyzed by quantitative real-time polymerase chain reaction. Results shown are from LCLs from 6 donors and are expressed as mean values  $\pm$  standard error of the mean. *P* values were calculated using a Wilcoxon signed rank test. \**P* < .05.

individuals in this study. Thus, enhanced EBV lytic replication was not due to a lower proinflammatory or higher antiinflammatory state of the tonsils.

Intriguingly, we found that heat-killed GAS and sonicated GAS exhibited contrasting effects on both EBV and immune activation. Whereas heat-inactivated GAS induced lytic EBV,

sonicated GAS did not. Sonicated GAS, by contrast, increased the expression of IL-6 in TMCs, whereas heat-inactivated GAS did not. Thus, heat-inactivated GAS—intact GAS bacterial cells that may therefore mimic GAS colonization—seem to activate the innate immunity differently from sonicated GAS and have an opposite influence on EBV. GAS DNA found in sonicated GAS suspensions triggers TLR9 and subsequently induces proinflammatory cytokine expression [18, 24]. Activation of Akata cells was diminished when a TLR9 antagonist was added (Supplementary Figure 2). This is compatible with our observation of increased IL-6 expression in TMCs exposed to sonicated GAS but not in TMCs exposed to heat-killed GAS. Importantly, we recently demonstrated that TLR9 triggering suppresses switching from latent to lytic EBV [15, 16].

The upregulation of *BZLF1* following exposure to heat-killed GAS was only approximately 1.5-fold but was highly reproducible, implying that only a small fraction of B cells was provoked to switch to lytic EBV infection. Indeed, we found that around 1% of LCL cells upregulated *BZLF1* expression after exposure to heat-killed GAS. An alternative reason for the increase in the level of EBV shedding in GAS-colonized children might be that they have an immunological difference that allows coincident GAS persistence and higher EBV secretion. Nevertheless, no such difference was observed for *IL-6*, *IL-10* (Figure 2B and 2C), and *TNF- $\alpha$*  mRNA expression levels (Supplementary Figure 3A). Surprisingly, heat-killed GAS did not induce switching to lytic EBV in Akata cells, which are commonly used as a model to study the switching from latent to lytic EBV. Thus, we hypothesized that Akata cells may differ from primary B cells and LCLs in their expression of TLRs. Indeed, we found that Akata cells expressed TLR2 at remarkably lower levels, if at all, than primary B cells and LCLs. Exposure of LCLs to GAS peptidoglycan, a known ligand for TLR2, provoked similar expression of *BZLF1* as heat-killed GAS. Upregulation of *BZLF1* was around 4-fold higher in TLR2-positive B cells, compared with TLR2-negative B cells. Importantly, anti-TLR2 neutralizing antibodies completely abrogated upregulation of *BZLF1* following exposure to heat-killed GAS or GAS peptidoglycan. This strongly suggested that heat-killed GAS and GAS peptidoglycan upregulated *BZLF1* expression in LCLs via TLR2 triggering. Finally, we showed that other known TLR2 ligands, namely staphylococcal lipoteichoic acid and peptidoglycan [28, 29], also induced *BZLF1* mRNA expression in LCLs, suggesting the engagement of TLR2 results in lytic reactivation of EBV.

Our novel findings imply that TLR2 triggering may disrupt EBV's default state of latency. This seems to be beneficial for EBV since it allows EBV to spread to other hosts. However, it may put EBV latency and the survival of the host cell at stake. In view that TLR2-expressing EBV-infected cells are likely to undergo switching to lytic EBV, selection of EBV-infected B-cells subsets with lower or no expression of TLR2 may take

place over time. Notably, we found that *TLR2* mRNA levels in EBV-negative BL cells were at least 5-fold higher than in EBV-positive BL cells (Supplementary Figure 3B), suggesting that cells expressing low TLR2 levels may have been more likely to survive than cells expressing higher levels of TLR2. Alternatively, EBV may induce downregulation of TLR2 expression, thereby preventing switching to lytic infection and securing its latent state. Indeed, the expression of *TLR2* in tonsillar B cells decreased in a time-dependent fashion after inoculation with EBV (Supplementary Figure 3C). Thus, TLR2 signal transduction may contribute to balancing latent and lytic EBV infection.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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**Potential conflict of interest.** All authors: No reports conflicts.

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